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Wake-promoting actions of noradrenergic α_1 - and β -receptors within the lateral hypothalamic area

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Abstract

Central norepinephrine exerts potent wake-promoting effects, in part through the actions of noradrenergic α_1 - and β -receptors located in the medial septal and medial preoptic areas. The lateral hypothalamic area (LHA), including the lateral hypothalamus, perifornical area and adjacent dorsomedial hypothalamus, is implicated in the regulation of arousal and receives a substantial noradrenergic innervation. To date the functional significance of this innervation is unknown. The current studies examined the degree to which noradrenergic α_1 - and β -receptor stimulation within the rat LHA modulates arousal. Specifically, these studies examined the wake-promoting effects of intra-tissue infusions (250 nL) of the α_1 -receptor agonist phenylephrine (10, 20 and 40 nmol) and the β -receptor agonist isoproterenol (3, 10 and 30 nmol) in rats. Results show that stimulation of LHA α_1 -receptors elicits robust and dose-dependent increases in waking. In contrast, β -receptor stimulation within the LHA had relatively modest arousal-promoting actions. Nonetheless, combined α_1 - and β -receptor stimulation elicited additive wake-promoting effects. Arousal-promoting hypocretin/orexin (HCRT)-synthesising neurons are located within the LHA. Therefore, additional immunohisto-chemical studies examined whether α_1 -receptor-dependent waking is associated with an activation of HCRT neurons as measured by FOS, the protein product of the immediate-early gene *c-fos*. Analyses indicate that although intra-LHA α_1 -receptor agonist infusion elicited a robust increase in FOS immunoreactivity (ir) in this region, this treatment did not activate HCRT neurons as measured by Fos-ir. Collectively, these observations indicate that noradrenergic α_1 -receptors within the LHA promote arousal via actions that are independent of HCRT neuronal activation.

Keywords

arousal; hypocretin/orexin; lateral hypothalamus; norepinephrine; sleep and wakefulness

Introduction

The regulation of arousal is an essential aspect of behavior and, ultimately, survival. Substantial evidence indicates prominent arousal-promoting actions of central noradrenergic systems (for review, Berridge, 2008). Seminal electrophysiological studies demonstrate that

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noradrenergic locus coeruleus (LC) neurons display low discharge rates during sleep and higher discharge rates during waking (Hobson *et al.*, 1975; Foote *et al.*, 1980; Aston-Jones & Bloom, 1981). Importantly, increases in LC discharge rates precede the transition to waking, suggesting a causal role of the LC–noradrenergic system in the induction of elevated arousal states (Foote *et al.*, 1980; Aston-Jones & Bloom, 1981). Consistent with this, selective activation of LC neurons elicits an increase in electroencephalographic and behavioral indices of arousal (Berridge & Foote, 1991; Carter *et al.*, 2010). Additional evidence demonstrates that the arousal-promoting actions of norepinephrine (NE) involve, at least in part, additive actions of α_1 - and β -receptors within two subcortical regions, the medial septal area (MSA) and the medial preoptic area (MPOA; for review, Berridge, 2008). Importantly, the noradrenergic innervation of the MSA and MPOA arises from not only the LC but also A1 and A2 noradrenergic nuclei (España & Berridge, 2006), suggesting that multiple noradrenergic systems exert arousal-promoting actions.

More recent evidence indicates a prominent role of the lateral hypothalamic area (LHA), including the lateral hypothalamus, perifornical area and adjacent dorsomedial hypothalamus, in the regulation of behavioral state. In particular, hypocretin/orexin (HCRT), synthesised solely by neurons located within the LHA, exerts potent wake-promoting effects (Peyron *et al.*, 1998; Sakurai *et al.*, 1998; España *et al.*, 2001; Sutcliffe & de Lecea, 2002; Adamantidis *et al.*, 2007). Interestingly, the LHA receives a moderately dense noradrenergic innervation from the LC and other noradrenergic nuclei (i.e. A1, A2; Baldo *et al.*, 2003; España *et al.*, 2005; Yoshida *et al.*, 2006). Surprisingly, despite extensive study of the behavioral state modulatory actions of both noradrenergic systems and the LHA, the functional significance of NE action within the LHA is largely unknown.

The current studies tested the hypothesis that noradrenergic α_1 - and β -receptors within the LHA act to promote waking. These studies demonstrate that microinfusion of the α_1 -receptor agonist phenylephrine (PHEN) into the LHA elicits large and dose-dependent wake-promoting actions. In contrast to the relatively robust wake-promoting effects of LHA α_1 -receptor activation, intra-LHA infusion of the β -agonist isoproterenol (ISO) had relatively modest wake-promoting effects. Under typical physiological conditions, NE release results in the simultaneous activation of α_1 - and β -receptors. Therefore, additional studies examined the wake-promoting actions of combined intra-LHA α_1 - and β -receptor agonists. These studies demonstrate an additive effect of α_1 - and β -receptors on waking. Finally, double-label immunohistochemical studies demonstrate that the wake-promoting effects of LHA α_1 -receptor stimulation are not associated with an activation of HCRT neurons, as measured by Fos-ir.

The current observations demonstrate that NE acts within the LHA to promote arousal. Moreover, when combined with previous studies these results indicate that NE-dependent waking involves actions at α_1 - and β -receptors within a distributed network of subcortical regions.

Materials and methods

Animals and surgery

Sixty-eight male Sprague–Dawley rats (260–280 g; Charles River, Wilmington, MA, USA) were housed in pairs with ad libitum access to food and water on an 11:13-h light:dark cycle (lights on 07.00 h). Under halothane anesthesia, 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA) were stereotactically implanted bilaterally, aimed at the lateral hypothalamic area (flathead; AP, –2.8; ML, $\pm$ 2.15; VD, –3.8 mm; at an angle of 6° from vertical). Additionally, an electroencephalographic (EEG) electrode was implanted into the frontal cortex (AP, +2.7; ML, 1.5 mm) and an electromyographic (EMG) electrode implanted into the dorsal neck muscle. All coordinates are relative to bregma. The cannulae, EEG and EMG electrodes were cemented into position using acrylic cement (Esschem, Linwood, PA, USA). A stainless-steel wire stylet was inserted and attached to each cannula via a threaded plastic connector (Plastics One). Animals recovered for 5–7 days before testing. All efforts were made to minimise the number of animals used. All facilities and procedures were in accordance with animal use and care guidelines put forth by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

Experimental procedures

On the day prior to testing, the stylet was removed and a mock infusion was conducted (33-gauge needle insertion). Rats were then transferred to sound-attenuated testing chambers and a stainless-steel coil spring was threaded onto the cannula via plastic threaded sleeves (Plastics One). The other end of the spring was attached to a counterbalance outside the chamber. Animals were housed individually overnight with free access to food and water.

On the morning of testing, between the hours of 09.00 and 11.00, a 33-gauge infusion needle was connected to PE20 tubing filled with water protected by a stainless-steel coil spring (Plastics One). The infusion needle was loaded with vehicle [artificial extracellular fluid (AECF); in mM: NaCl, 147; CaCl₂, 1.3; MgCl₂, 0.9; and KCl, 2.5] or drug dissolved in AECF with a 50-nL air bubble separating drug or vehicle from water. The infusion needle was inserted into the cannula, extending 4.0 mm beyond the end, and secured. EEG and EMG electrodes were connected to a field-effect transistor headstage. Baseline EEG and EMG recordings were initiated 1–2 h following needle insertion when the animal had returned to quiet rest or sleep. Infusions of 250 nL (125 nL/min) were made, following at least 1 h in which the majority of the time was spent asleep, using a microprocessor-controlled pump (Harvard Apparatus, South Natick, MA, USA). This infusion volume was based on previous studies that demonstrated that although 150-nL infusions of the α_1 - and β -agonists elicited consistent waking when infused into the MSA (Berridge & Foote, 1996), 250-nL infusions were required when infused into the MPOA (Berridge & O'Neill, 2001). Given that the LHA is no smaller than the MPOA, the current studies used 250-nL infusion volume. Limited pilot studies confirmed that this infusion volume provided consistent behavioral effects when infused into the LHA while minimising behavioral effects when infusions were placed outside the LHA. See below for further discussion of the suitability of

this infusion volume for these studies. Behavioral data were collected continuously onto polygraph and video recording tape for at least 2 h following infusion.

Drugs

The β -receptor agonist ISO (Sigma, St Louis, MO, USA) and the α_1 -receptor agonist PHEN (Sigma) were dissolved in AECF. Unilateral infusions were used except where otherwise stated. Doses were based on previous results obtained with infusions of these drugs into the MSA and MPOA (Berridge & Foote, 1996; Berridge & O'Neill, 2001) as well a limited number of pilot studies.

EEG and EMG recordings and analyses

Cortical EEG and EMG were recorded as previously described (Berridge & Foote, 1996). EEG and EMG traces were hand-scored for the following behavioral states: (i) slow-wave sleep (high-voltage EEG, low-voltage EMG); (ii) rapid-eye movement (REM) sleep (low-voltage EEG, EMG activity $\sim 50\%$ lower amplitude than that observed in slow-wave sleep, with occasional short-duration, large-amplitude deflections due to muscle twitches); (iii) waking (low-voltage EEG, sustained high-voltage EMG of an average amplitude at least twice that observed in slow-wave sleep, often with frequent large-amplitude movement deflections; see Fig. 1). To be scored as a distinct state, the EEG and EMG activity patterns needed to persist for a minimum of 15 s.

Time spent in each state was scored for each 30-min epoch. These included two epochs immediately prior to infusions (PRE 1, 0–30 min; PRE 2, 30–60 min) and three epochs immediately following infusions (POST 1, 0–30 min; POST 2, 30–60 min; POST 3, 60–90 min). EEG and EMG scoring was conducted by an observer blind to experimental conditions.

Histology and data selection

Rats were deeply anesthetised and perfused transcardially with 4% formaldehyde. The brain was removed and placed in fixative for at least 24 h, frozen, and 40- μ m sections through the LHA collected. The location of the infusion site was determined from Nissl-stained sections. Data were included in the analyses only when both the EEG and EMG recordings were adequate and the histological analyses verified accurate placements of infusion needles (Fig. 1).

Immunohistochemistry

A separate group of animals ($n = 5$) simultaneously received intra-LHA infusions of 20 nmol PHEN into one hemisphere and vehicle into the contralateral hemisphere. Following a 90-min EEG–EMG recording period, animals were deeply anesthetised with pentobarbital and perfused transcardially with 250 mL heparinised saline (1 unit of heparin per mL of 0.9% saline; Baxter Healthcare Corporation, Deerfield, IL, USA) followed by 400 mL of 4% formaldehyde in 0.01 M phosphate buffer. Brains were removed, stored in formal-dehyde overnight and taken through graded sucrose solutions (20–30%). Forty-micrometer-thick sections were collected and placed in 0.01 M phosphate-buffered saline (PBS) with 0.1% sodium azide and stored at 4 °C. Sections were first stained for Fos [with diaminobenzidine

(DAB); brown reaction product] and then counter-stained for prepro-HCRT (Vector SG; blue reaction product; Vector Laboratories, Burlingame, CA, USA). Prepro-HCRT is a precursor protein that gives rise to both HCRT-1 and HCRT-2 neuropeptides.

For immunohistochemical processing of Fos-ir, sections were rinsed with 0.01 M PBS, incubated for 20 min in a quench solution containing 0.75% hydrogen peroxide, rinsed again and transferred for 30 min to a blocking solution containing 10% normal goat serum (NGS) and 0.3% Triton X-100 in 0.01 M PBS. Sections were then rinsed and incubated for 48 h at 4 °C with rabbit anti-rat Fos antibody (1 : 20 000; Oncogene, San Diego, CA, USA) diluted in 0.01 M PBS containing 0.4% NGS. After incubation, tissue was rinsed with 0.01 M PBS and incubated with biotinylated goat anti-rabbit antibody (1 : 200; ABC kit; Vector Laboratories) for 90 min. Tissue was then rinsed with 0.01 M PBS, exposed to an avidin–biotin peroxidase complex (ABC complex; Vector Laboratories) for 60 min and rinsed with 0.01 M PBS. Tissue was then reacted for Fos with DAB (Vector Laboratories) to yield a brown precipitate.

For immunohistochemical processing of prepro-HCRT, sections were then rinsed with 0.3% Triton X-100 in 0.01 M PBS and then quenched and blocked as describe above. Sections were then rinsed and incubated for 24 h at 4 °C with rabbit anti-rat prepro-HCRT antibody (1 : 2000; Chemicon International, Temecula, CA, USA) diluted in 0.1 M PBS containing 0.1% Triton X-100. Tissue was then rinsed with 0.3% Triton X-100 in 0.01 M PBS and incubated with biotinylated goat anti-rabbit secondary antibody (1 : 200; ABC kit; Vector Laboratories) for 90 min, rinsed again and then counterstained with Vector SG (Vector Laboratories) to yield a blue–gray precipitate.

After staining, sections were mounted on microscope slides (Fisher Scientific, Itasca, IL, USA), dehydrated, cleared (Histoclear; Fisher Scientific) and coverslipped.

Immunohistochemistry analyses

Single-labeled-ir Fos-immunoreactive (-ir) nuclei, single-labeled prepro-HCRT-ir neurons and double-labeled prepro-HCRT-ir + Fos-ir neurons were analysed by examiners blind to treatment conditions from digital images captured by a Zeiss Axiocam digital video camera connected to an Olympus BX51 microscope. Images were collected using horizontal capture-windows with dimensions of $\sim 300 \times 400 \mu\text{m}$ (400 $\times$ magnification). Cell counts for each hemisphere were derived from eight 400 $\times$ capture-windows (Fig. 6). The windows were placed to span nearly the entire population of prepro-HCRT-ir neurons within a coronal section. This process was performed on three coronal sections per hemisphere taken at different levels across the anterior–posterior extent of the HCRT field (AP ~ -2.00 , -2.45 and -2.85 mm). Counts were averaged across these three sections. Cell counts were excluded for portions of a capture window containing needle-related tissue damage. In general, needle damage represented a small percentage of the portion of tissue excluded from analyses ($2.1 \pm 0.6\%$). For prepro-HCRT-ir neurons, only cells containing a distinct soma with blue precipitate were counted in order to minimise double-counting labeled neurons. Similarly, for Fos-ir + prepro-HCRT-ir double-label analyses, cells were counted only when an obvious nucleus containing brown precipitate was contained within a distinct soma containing blue precipitate (Fig. 7).

Statistical analyses

EEG–EMG-based measures of sleep–wake state were analysed statistically with a two-way mixed-design ANOVA with drug treatment as the between-subjects variable and time as the within-subject variable. Where between-subjects omnibus tests proved significant, pairwise post hoc group comparisons for each time epoch were made using a one-way ANOVA. In the case of the combined agonists (PHEN and ISO), a one-way ANOVA was used to analyse waking in the first 30-min time epoch (POST 1) following drug or vehicle infusion. Post hoc analyses were conducted with Tukey's honestly significant differences test. Cell counts (single-labeled Fos-ir and prepro-HCRT-ir neurons, and percentage of double-labeled Fos-ir + prepro-HCRT-ir neurons) were analysed statistically with a within-subject non-directional t-test between vehicle- and drug-treated hemispheres.

Results

Effects of α_1 -receptor stimulation within LHA on waking

To assess the wake-promoting effects of LHA α_1 -receptor stimulation, rats received 250 nL intra-LHA infusions of either vehicle ($n = 8$) or various doses of the α_1 -receptor agonist PHEN: 10 nmol ($n = 8$), 20 nmol ($n = 15$) and 40 nmol ($n = 8$). All infusions were unilateral except for an additional group receiving bilateral 20 nmol PHEN ($n = 8$). Animals spent the majority of time asleep prior to infusions under these testing conditions. No significant differences in time spent awake or asleep were observed prior to or following vehicle infusions into the LHA (see Fig. 2).

Intra-LHA PHEN elicited a dose-dependent increase in time spent awake (Fig. 2; treatment, $F_{4,42} = 16.56$, $P < 0.001$; time, $F_{4,168} = 52.89$, $P < 0.001$; treatment $\times$ time, $F_{16,168} = 6.01$, $P < 0.001$) and decreases in time spent in slow-wave sleep and REM sleep (slow-wave sleep: treatment, $F_{4,42} = 9.75$, $P < 0.001$; time, $F_{4,168} = 46.46$, $P < 0.001$; treatment $\times$ time, $F_{16,168} = 6.25$, $P < 0.001$; REM sleep: treatment, $F_{4,42} = 6.54$, $P = 0.001$; time, $F_{4,168} = 7.24$, $P < 0.001$; treatment $\times$ time, $F_{16,168} = 1.36$, $P = 0.17$). At 20 nmol, the average latency for PHEN-induced waking was 253 ± 79 s from the end of the 120-s infusion. The wake-promoting actions of PHEN followed an inverted-U dose–response curve, such that time spent awake was significantly increased during the POST 1 epoch in the 20-nmol PHEN group (1228 ± 103) but not in the 10-nmol group (612 ± 83) or the 40-nmol group (659 ± 86) relative to vehicle treatment (281 ± 45). During the POST 2 epoch, time spent awake was significantly increased in the 40-nmol group (977 ± 140). When infused bilaterally, 20 nmol PHEN elicited larger increases in waking and decreases in slow-wave and REM sleep than seen with unilateral infusions (Fig. 2).

Additional mapping studies were conducted with 20 nmol PHEN, a dose that elicited robust increases in waking. As shown in Fig. 3, PHEN-induced waking was observed with infusions within the majority of the medial–lateral extent of the LHA (including the lateral hypothalamus proper, perifornical area and adjacent dorsomedial hypothalamus) known to contain neurons implicated in the modulation of sleep/wake state (i.e. HCRT- and melanin-concentrating hormone-synthesising neurons). In contrast, infusions placed outside of this region (i.e. anterior and posterior, as well as dorsal, ventral and lateral, to the LHA) had

minimal or less consistent effects on waking, with time spent awake similar to that seen in vehicle-treated animals (Fig. 3).

Effects of β -receptor stimulation within LHA on waking

To assess the wake-promoting effects of LHA β -receptor stimulation, rats received unilateral intra-LHA infusions (250 nL) of vehicle ($n = 8$) or varying doses of the β -receptor agonist ISO: 3 nmol ($n = 9$), 10 nmol ($n = 8$) or 30 nmol ($n = 9$). ISO infusion elicited a modest, though statistically significant, increase in time spent awake (Fig. 4; treatment, $F_{3,30} = 3.71$, $P = 0.022$; time, $F_{4,120} = 14.47$, $P < 0.001$; treatment $\times$ time, $F_{12,120} = 1.94$, $P = 0.03$) and a decrease in REM sleep (treatment, $F_{3,30} = 8.18$, $P < 0.001$; time, $F_{4,120} = 0.95$, $P = 0.439$; treatment $\times$ time, $F_{12,120} = 0.98$, $P = 0.469$). There was a non-significant trend for a decrease in slow-wave sleep (treatment, $F_{3,30} = 0.72$, $P = 0.546$; time, $F_{4,120} = 13.57$, $P < 0.001$; treatment $\times$ time, $F_{12,120} = 1.78$, $P = 0.060$). At 10 nmol, the average latency for ISO-induced waking was 654 ± 181 s from the end of the infusion. At 30 nmol, ISO elicited a modest but significant increase in time spent awake in the POST 1 epoch. The 10-nmol dose produced a larger increase in waking, but this was delayed until the POST 2 epoch. Infusions of ISO placed outside the LHA failed to promote waking ($n = 4$; data not shown).

Effects of combined α_1 - and β -receptor stimulation within LHA on waking

Under physiologically relevant conditions, NE acts simultaneously at α_1 - and β -receptors. Therefore, additional experiments examined the degree to which the wake-promoting actions of α_1 - and β -receptors are additive (Fig. 5). In this study, animals received an intra-LHA infusion containing both PHEN and ISO ($n = 8$) at doses that individually produced modest increases in waking (10 nmol each). Results were compared with those of vehicle treatment ($n = 8$) or each drug alone (PHEN, $n = 8$; ISO, $n = 8$).

As displayed in Fig. 5, the combined infusion of PHEN and ISO produced a larger and statistically significant increase in waking at POST 1 than seen with either drug alone (Fig. 5; treatment, $F_{3,31} = 9.93$, $P < 0.001$). The wake-promoting effects of combined PHEN–ISO infusion appeared largely additive. For example, combined PHEN–ISO resulted in 1027 s of waking compared to 612 and 634 s with PHEN and ISO administered individually, respectively. The average latency for waking from the end of the intra-LHA infusion was considerably shorter for the combined PHEN–ISO treatment than for treatment with either drug individually (combined PHEN–ISO, 87 ± 34 s; 10 nmol PHEN, 437 ± 94 s; 10 nmol ISO, 654 ± 181 s).

Effects of intra-LHA α_1 -receptor stimulation on HCRT neuronal activity

Immunohistochemical studies were conducted to assess whether the wake-promoting actions of LHA α_1 -receptors are associated with an activation of prepro-HCRT-ir neurons (as revealed by Fos-ir). In these studies, animals ($n = 5$) simultaneously received intra-LHA infusions of a wake-promoting dose of PHEN (20 nmol) into one hemisphere and vehicle into the contralateral hemisphere. All animals displayed prominent increases in time spent awake following intra-LHA infusions. PHEN infusion elicited a significant increase in overall Fos-ir within the LHA compared to vehicle-treated hemispheres (see Table 1; total, $t_4 = 3.15$, $P < 0.05$). Significant PHEN-induced increases in Fos-ir occurred in both the medial

and lateral subregions of the HCRT field (medial, $t_4 = 2.98$, $P < 0.05$; lateral, $t_4 = 4.75$, $P < 0.01$), with a strong trend for an increase in FOS-ir in the perifornical subregion ($t_4 = 2.46$, $P = 0.07$). However, PHEN did not increase the percentage of double-labeled neurons (i.e. number of double-labeled Fos-ir + prepro-HCRT-ir neurons divided by the total number of prepro-HCRT-ir neurons) in any these regions (see Table 1; total, $t_4 = 0.61$, $P = 0.57$; medial, $t_4 = 0.51$, $P = 0.64$; perifornical, $t_4 = 0.71$, $P = 0.52$; lateral, $t_4 = 0.72$, $P = 0.51$). There was no significant difference between the vehicle- and PHEN-treated hemispheres in the total number of prepro-HCRT-ir neurons counted (see Table 1; total, $t_4 = 2.28$, $P = 0.09$; medial, $t_4 = 2.06$, $P = 0.11$; perifornical, $t_4 = 2.07$, $P = 0.11$; lateral, $t_4 = 1.13$, $P = 0.32$).

Discussion

Central noradrenergic systems exert potent arousal-promoting actions (for review see Berridge, 2008). Prior work demonstrated that NE-induced arousal involves additive actions of α_1 - and β -receptors within two subcortical regions, the MSA and MPOA. More recently, the LHA has been identified as playing a prominent role in the regulation of arousal (Peyron *et al.*, 1998; Sakurai *et al.*, 1998; España *et al.*, 2001; Alam *et al.*, 2002; Sutcliffe & de Lecea, 2002; Adamantidis *et al.*, 2007). Given the key role played by both the LHA and noradrenergic systems in the regulation of arousal, it is surprising that the behavioral state modulatory actions of noradrenergic signaling within the LHA had not been examined previously. The current observations provide the first evidence that NE α_1 - and β -receptors act within the general region of the LHA to promote waking. Additional immunohistochemical studies indicate that the wake-promoting actions of LHA noradrenergic α_1 - receptors do not involve HCRT neuronal activation, consistent with prior *in vitro* electrophysiological studies (Yamanaka *et al.*, 2003; Li & van Den Pol, 2005). Collectively, the current and previous studies demonstrate that the arousal-promoting actions of noradrenergic systems involve NE signaling at α_1 - and β -receptors in an extended network of subcortical regions that include the MSA, MPOA and LHA.

Wake-promoting actions of LHA α_1 - and β -receptors

Across the hemispheres, the wake-promoting actions of α_1 -receptors within the LHA were largely additive, with bilateral α_1 -receptor agonist infusions resulting in larger increases in time spent awake than unilateral infusions. This suggests that extended circuitry activated by intra-LHA infusions is largely segregated across the hemispheres and that this extended circuitry elicits additive wake-promoting actions. Given that projections of LHA HCRT neurons to arousal-related structures are primarily ipsilateral in nature (España *et al.*, 2005), it is likely that the wake-promoting actions of LHA NE involve activation of extended circuitry in an ipsilateral manner.

The wake-promoting effects of LHA α_1 - and β -receptors were also additive. The cellular mechanisms responsible for the additive actions of LHA α_1 - and β -receptors are currently not known. It is possible that these receptors are located on the same cell, and that stimulation of α_1 -receptors enhances the second-messenger actions of β -receptors, as has been observed in other brain regions (Stone & Herrera, 1986; Petitti & Etgen, 1991). Alternatively, β -receptors may potentiate the wake-promoting actions of α_1 -receptors within

the LHA. This latter mechanism could explain the relatively weak wake-promoting effects of LHA β -receptor stimulation observed in the current studies, given that NE release is minimal under the resting or sleeping baseline conditions used in the current studies. Additionally or alternatively, additive effects may involve α_1 - and β -receptors located on different cell populations, each positively coupled to waking. To date, the degree to which noradrenergic receptors are anatomically and neurochemically segregated in the LHA is largely unknown, representing an important topic for future research.

Methodological considerations

The current studies utilised small intra-LHA infusions (250 nL) of α_1 - and/or β -receptor agonists to investigate the arousal-promoting actions of LHA noradrenergic neurotransmission. A critical issue with the use of intra-tissue infusions is identification of the anatomical boundaries associated with infusion-induced alterations in behavior. Prior studies demonstrate that differing chemical properties can display varying diffusion properties, thus preventing use of a standard model of diffusion (see Berridge *et al.*, 1993 vs. Berridge & Foote, 1991). Moreover, although radioactive labeling can be used to measure drug diffusion in tissue, this approach suffers from the fact that sensitivity for detecting radioactivity can greatly exceed physiologically-relevant levels. For example, detection in tissue of radiolabeled carbachol was observed at 1/10 000th the concentration demonstrated to elicit behavioral effects (Gnadt & Pegram, 1986). At this dilution, our infusions would not exert behaviorally-relevant actions. Therefore, we believe that mapping studies are an essential tool for unambiguously determining the site of action of a drug.

Infusion volume is an important issue when conducting mapping studies. Prior work with the same drugs used in the current study indicated that, although consistent wake-promoting effects were observed with 150 nL infusions into the MSA (Berridge & Foote, 1996; Berridge *et al.*, 1996), a larger volume of 250 nL was required for the larger volume of tissue in the MPOA (Berridge & O'Neill, 2001). Given that the LHA also consists of a relatively large volume of tissue, the current studies used the larger infusion volume of 250 nL. The current mapping studies indicated that this infusion volume provides a radius of action of 500 μ m, consistent with our previous observations. Thus, as shown in Fig. 3, infusions placed 300–500 μ m outside the identified region associated with wake-promoting actions (e.g. into the ventromedial hypothalamus or zona incerta) failed to elicit robust waking. The ability of 250 nL infusions to exert anatomically restricted actions probably reflects in part the fact that, as the radius of a sphere expands, the volume increases directly with the cube of the radius. Therefore, following intra-tissue infusion of a drug, there will be a sharp drop-off in concentration as a drug diffuses radially from the infusion site. This decrease in drug concentration has been confirmed with more realistic models of drug diffusion in tissue (i.e. Newton's inverse-square law and Crank–Nicholson method; Devilbiss *et al.*, 2012). Collectively, these observations indicate that the 250 nL infusion volume used in these studies is sufficient for delineating anatomical boundaries within the LHA for the wake-promoting actions of an α_1 -receptor agonist.

The results of the current studies demonstrate that NE action at both α_1 - and β -receptors within the LHA is sufficient to promote waking. An additional question of interest is

whether LHA NE is necessary for normal arousal or waking. However, the modulation of arousal or waking involves a number of redundant systems (Vanderwolf & Robinson, 1981; Pape & McCormick, 1989; Berridge & Waterhouse, 2003). As such, inactivation of a single wake-promoting system often has minimal effects on overall arousal state. This is even more true for inactivation of NE within a single terminal field, given that multiple terminal fields are associated with noradrenergic modulation of arousal. Therefore, as we have observed for other regions associated with NE-induced arousal (e.g. MSA; Berridge & Wifler, 2000), we predict that blocking noradrenergic receptors within the LHA will have minimal effects on arousal state. Nonetheless, this is an important topic for future studies.

Finally, it is important to note that the focus of the current studies was on the behavioral actions of noradrenergic receptors within the LHA. Although there are newer techniques (e.g. optogenetics) that are useful for activating and suppressing neurochemically defined neurons, these are not yet useful for selectively activating specific receptors (e.g. NE α_1 -receptors; for review, see Yizhar *et al.*, 2011). Thus, currently, intra-tissue infusions of highly selective and well characterised receptor ligands are best suited for the current research questions.

Intra-LHA α_1 -receptor agonist-induced waking did not involve activation of HCRT neurons

HCRT neurons located within the LHA exert prominent arousal-enhancing actions. Previous *in vitro* whole-cell patch-clamp studies demonstrated that NE inhibits, rather than excites, HCRT neuronal activation (Yamanaka *et al.*, 2003, 2006; Li & van Den Pol, 2005). However, limited evidence suggests that this noradrenergic inhibitory action is α_2 -receptor-dependent (Li & van Den Pol, 2005) and may be restricted to sleep deprivation conditions (Uschakov *et al.*, 2006). Moreover, the neuromodulatory actions of NE are probably highly dependent on the activity state of a neuron and thus *in vitro* conditions may not accurately reflect the actions of a transmitter *in vivo* (Berridge & Waterhouse, 2003). Therefore, in the current studies we examined whether the wake-promoting actions of α_1 -receptors involve an activation of HCRT neurons within the LHA, as revealed by Fos-ir. Although Fos is not a perfect indicator of neuronal activity, Fos-based indices of HCRT neuronal activity across varying conditions closely match measures of peptide release and electrophysiological recordings of HCRT neuronal discharge (España *et al.*, 2003; Mileykovskiy *et al.*, 2005). Thus, Fos appears to be an accurate indicator of HCRT neuronal activity under varying conditions.

These studies demonstrate that the wake-promoting effect of α_1 -receptor activation was not associated with concurrent activation of HCRT neurons. This observation does not rule out a potential excitatory action of NE on HCRT neurons under normal physiological conditions. For example, as discussed above, endogenously released NE will stimulate both α_1 - and β -receptors within the LHA. Thus, it is possible that the combined action of these receptors could activate HCRT neurons (Estabrooke *et al.*, 2001; Mileykovskiy *et al.*, 2005; Berridge *et al.*, 2010). Moreover, this observation could be consistent with limited observations indicating that as few as 16% of HCRT neurons express α_1 -receptors (Modirrousta *et al.*, 2005). Nonetheless, the current studies suggest that α_1 -receptors within the LHA promote arousal via a mechanism that is independent of HCRT neuronal activation.

In addition to HCRT neurons, the LHA also contains melanin-concentrating hormone (MCH)-synthesising neurons implicated in the promotion of sleep (Verret *et al.*, 2003; for review, see Adamantidis & de Lecea, 2008). Consistent with this, limited evidence suggests that LHA MCH neurons probably act as sleep-active neurons (Modirrousta *et al.*, 2005). Interestingly, in vitro studies demonstrate that NE inhibits MCH neuronal activity (Bayer *et al.*, 2005). Similarly, NE was observed to inhibit sleep-active neurons in the MPOA, albeit through α_2 -receptors (Osaka & Matsumura, 1995). Thus, the wake-promoting actions of NE within the dorsomedial LHA may involve inhibition of MCH neuronal signaling. The cellular targets involved in the wake-promoting (or sleep-inhibiting) actions of NE within the LHA are an important topic for future studies.

Finally, the majority of LHA NE arises from the A1 and A2 noradrenergic nuclei (Yoshida *et al.*, 2006), which are known to express the arousal-promoting prolactin-releasing peptide (PrRP; Chen *et al.*, 1999; Lin *et al.*, 2002). Given this, co-release of PrRP may act additively with NE in the LHA to activate HCRT or MCH neurons. This is an important direction for future research. Moreover, DMH neurons in particular exhibit PrRP immunoreactivity (Maruyama *et al.*, 1999); thus it is possible that NE action within the LHA may modulate PrRP neuronal activity to modulate sleep–wake states.

Neurocircuitry of NE-induced arousal

Prior work in our laboratory has demonstrated that NE acts within the MSA and MPOA to promote arousal (for review, see Berridge, 2008). Although the MSA and MPOA receive the majority of their noradrenergic innervation from the LC (~50%), the remainder arises from other noradrenergic nuclei, particularly A1 (~25%) and A2 (~25–40%), suggesting a prominent wake-promoting action of multiple noradrenergic nuclei (España & Berridge, 2006). This conclusion is consistent with anatomical evidence indicating that, while the LC projects to the LHA, the vast majority of noradrenergic input to this region arises from outside the LC (~80–95%; Yoshida *et al.*, 2006). In addition to these subcortical sites, in vitro NE acts within the cortex and thalamus to induce firing patterns associated with waking or arousal (McCormick *et al.*, 1991). Collectively, these observations indicate that NE, arising from multiple noradrenergic nuclei, acts within a distributed network of cortical and subcortical sites to promote waking/arousal.

Limited observations suggest that there may be a differential sensitivity to the wake-promoting actions of noradrenergic receptor activation across subcortical regions. Specifically, the magnitude of waking following stimulation of LHA α_1 -receptors was ~30% smaller than that seen with α_1 -receptor activation in the MSA and MPOA (for review, see Berridge, 2008). There are at least two potential explanations for the reduced sensitivity of the wake-promoting actions of LHA α_1 -receptor stimulation relative to the MPOA and MSA. First, the general region of the LHA associated with NE receptor agonist-induced waking is comparatively large. Thus, it is possible that the relatively small infusion volume (250 nL) used in these studies resulted in drug diffusion throughout a smaller percentage of the LHA than that seen for the MSA or MPOA. Alternatively, activation of LHA NE receptors may result in less potent activation of arousal-related circuits. The differential sensitivity to wake-promoting actions of α_1 -receptors across these sub-cortical regions

(MPOA and MSA vs. LHA) appears to align with different sources of noradrenergic afferents to these regions reviewed above – the majority of the NE innervation of the MSA and MPOA arises from the LC while the majority of the LHA NE innervation arises from outside the LC. Therefore it is possible that, although noradrenergic nuclei outside the LC contribute to the modulation of arousal, they are less effective in these actions.

Summary

These studies provide the first demonstration that NE acts within the LHA to promote waking via actions of α_1 - and β -receptors. Despite the prominent arousal-promoting actions of HCRT neurons, the wake-promoting effects of intra-LHA α_1 -receptor agonist infusion were not associated with an activation of HCRT neurons as revealed by Fos-ir.

These observations provide new understanding regarding the neurocircuitry involved in the regulation of behavioral state as well as the neurobiology of both the LHA and central noradrenergic systems.

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Abbreviations

AECF	artificial extracellular fluid
EEG	electroencephalographic
EMG	electromyographic
HCRT	hypocretin/orexin
ir	immunoreactivity
-ir	-immuno-reactive
ISO	isoproterenol
LC	locus coeruleus
LHA	lateral hypothalamic area
MCH	melanin concentrating hormone
MPOA	medial preoptic area
MSA	medial septal area
NE	norepinephrine
PBS	phosphate-buffered saline
PHEN	phenylephrine
REM	rapid eye movement

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Fig. 1.

(Left panel) Photomicrograph depicting placement of the infusion needle within the LHA. Shown is the ventral-most portion of the needle track located within the LHA. Arrow indicates ventral-most extent of needle track. (Right panel) Raw cortical EEG and EMG traces are displayed. Shown are 5-min recordings of EEG and EMG prior to (Pre-Infusion) and beginning 2 min following the end of (Post-Infusion) intra-LHA infusion of 20 nmol PHEN. Prior to the infusion the animal spent the majority of time asleep, reflected in large-amplitude slow-wave EEG activity combined with low-amplitude EMG activity. Following PHEN infusion, the animal spent the majority of the time awake, reflected in a decrease in EEG amplitude and an increase in EMG amplitude, including large deflections indicative of active movement. 3V, third ventricle; fx, fornix; opt, optic tract.

Fig. 2.

Effects of intra-LHA infusion of α_1 -receptor agonist PHEN on EEG- and EMG-defined sleep-wake states. Shown are the dose-dependent effects of unilateral infusion of vehicle or 10, 20 or 40 nmol PHEN, and bilateral infusion of 20 nmol PHEN into the LHA on time spent in waking, slow-wave sleep and REM sleep states. Symbols represent mean ($\pm$ SEM) time (s) spent awake per 30-min epoch. PRE 1 and PRE 2 represent pre-infusion epochs. POST 1–POST 3 represent post-infusion epochs. * $P < 0.05$, ** $P < 0.01$ compared to vehicle-treated animals.

Fig. 3.

Schematic depiction of the location of unilateral intra-LHA infusion of 20 nmol PHEN and the amount of time spent awake associated with each infusion. Symbols indicate the position of the ventral tip of the infusion needle determined from histological sections. Numerals specify the range for time spent awake in the first 60 min following the infusion (1, 0–900 s; 2, 901–1800 s; 3, 1801–2700 s; 4, 2701–3600 s). Panels are arranged anterior to posterior with the anterior-most panel shown in the upper left and the posterior-most panel in the bottom right position. Vehicle-treated rats displayed a mean time awake in this period of 736.94 s (a numeric value of 1), reflecting the fact that typically animals will spontaneously wake for relatively brief periods to groom, eat and drink and then return to sleep. Dashed lines identify a region within which PHEN infusion promoted waking. This region encompasses the lateral hypothalamus, the perifornical area and the adjacent dorsomedial hypothalamus (collectively referred to as LHA). Infusions outside of this general region (i.e. dorsal, ventral and lateral to LHA and the far caudal extent) were substantially less effective at eliciting waking beyond levels observed in either untreated pre-infusion epochs or following vehicle treatment (i.e. < 1200 s, assigned numeric values of 1 and 2). This α_1 -receptor agonist-sensitive region primarily aligns with the region known to contain neurons implicated in the modulation of sleep–wake state (i.e. HCRT and MCH neurons). The only exception to this was one case in which infusion of the PHEN into the zona incerta elicited waking (AP –2.00 mm; Swanson, 1992). In this case, however, the infusion was in close proximity to the LHA. Thus, it is likely that the waking observed in this case reflects drug

diffusion into the LHA. AHN, anterior hypothalamic nucleus; ARH, arcuate nucleus hypothalamus; cpd, cerebral peduncle; DMH, dorsomedial nucleus hypothalamus; fx, columns of the fornix; GP, globus pallidus; LHA, lateral hypothalamic area; mtt, mammillothalamic tract; opt, optic tract; PH, posterior hypothalamic nucleus; pm, principal mammillary tract; V3, third ventricle; VM, ventral medial nucleus of the thalamus; VMH, ventromedial nucleus of the hypothalamus; VPL, ventral posterolateral nucleus of the thalamus; VPM, ventral posteromedial nucleus of the thalamus; ZI, zona incerta.

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Fig. 4.

Effects of intra-LHA infusion of the β -receptor agonist ISO on EEG- and EMG-defined sleep-wake states. Shown are the dose-dependent effects of vehicle and 3, 10 and 30 nmol ISO infused unilaterally into the LHA on time spent in waking, slow-wave sleep and REM sleep states. Symbols represent mean ($\pm$ SEM) time (s) spent awake per 30-min epoch. PRE 1 and PRE 2 represent pre-infusion epochs. POST 1–POST 3 represent post-infusion epochs. * $P < 0.05$, ** $P < 0.01$ compared to vehicle-treated animals.

Fig. 5.

Effects of combined intra-LHA infusion of α_1 - and β -receptor agonists (PHEN and ISO, respectively) on EEG- and EMG-defined waking state. Shown are the effects of vehicle, 10 nmol PHEN, 10 nmol ISO, and 10 nmol PHEN + 10 nmol ISO (PHEN/ISO) intra-LHA infusions on time spent in the waking state. Bars display mean ($\pm$ SEM) time (s) spent awake in the first 30-min epoch following infusion (POST 1). ** $P < 0.01$ compared to vehicle-treated animals.

Fig. 6.

Photomicrograph (captured at 40 $\times$, cropped to $\sim$ 80 $\times$) depicting the distribution of prepro-HCRT-ir neurons within the LHA. Boxes represent approximate locations of eight capture-windows used to collect 400 $\times$ digital images for single-labeled Fos-ir, prepro-HCRT-ir and double-labeled Fos-ir + prepro-HCRT-ir cell counts. Cell counts were summed for all boxes (1–8) as well as for the medial (1–4), perifornical (4 and 6) and lateral (5–8) subregions (see Table 1). 3V, third ventricle; fx, fornix; mmt, mammillothalamic tract; opt, optic tract. Scale bar, 250 μ m.

Fig. 7. Photomicrographs depicting Fos-ir nuclei (brown) and prepro-HCRT-ir neurons (blue) within the LHA following intra-LHA infusion of vehicle into one hemisphere and 20 nmol PHEN into the contralateral hemisphere of the same animal. (Top panel) Photomicrograph (400 $\times$) of the vehicle-treated hemisphere; note modest number of Fos-ir nuclei. (Middle panel) Photomicrograph (400 $\times$) of the PHEN-treated hemisphere. (Bottom panel) Photomicrograph (1000 $\times$) of boxed region indicated in the middle (PHEN-treated) panel. Arrows indicate examples of HCRT-ir neurons. The asterisk indicates a double-labeled cell that is present in both the middle and bottom panels. Intra-LHA infusion of 20 nmol PHEN significantly increased the number of Fos-ir nuclei as compared to vehicle-treated

hemispheres (see Table 1). Scale bars, 125 μm (top and middle panels), 50 μm (bottom panel).

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Table 1.

Number of FOS-ir-labeled nuclei, prepro-HCRT-ir-labeled neurons, and the percentage of LHA neurons double-labeled for FOS-ir + prepro-HCRT-ir in vehicle- and PHEN-treated hemispheres

	Total (boxes 1–8)			Medial (boxes 1–4)			Perifornical (boxes 4 and 6)			Lateral (boxes 5–8)		
	Fos-ir	HCRT-ir	% DBL	Fos-ir	HCRT-ir	% DBL	Fos-ir	HCRT-ir	% DBL	Fos-ir	HCRT-ir	% DBL
Vehicle (AECF)	585.1 ± 120.5	68.3 ± 10.2	15.6 ± 7.4	258.1 ± 60.6	46.7 ± 6.7	12.2 ± 6.3	158.4 ± 48.0	23.8 ± 4.0	19.4 ± 9.8	246.4 ± 57.6	24.9 ± 4.8	19.2 ± 11.9
PHEN (20 nmol)	968.5 ± 196.8 *	47.5 ± 8.7	10.4 ± 2.8	526.5 ± 104.1 *	30.8 ± 5.8	8.8 ± 3.5	207.4 ± 49.8	15.3 ± 2.1	12.0 ± 4.4	373.3 ± 81.2 **	22.1 ± 3.3	10.1 ± 3.4

Mean ± SEM number of FOS-ir-labeled nuclei (FOS-ir), prepro-HCRT-ir-labeled (HCRT-ir) neurons and the percentage of double-labeled neurons (% DBL; i.e. number of double-labeled FOS-ir + prepro-HCRT-ir neurons as a percentage of the total number of prepro-HCRT-ir neurons) in vehicle- and 20 nmol PHEN-treated hemispheres across various subregions (total, medial, perifornical and lateral) of the HCRT field. Box numbers refer to capture windows displayed in Fig. 6. All cells counts were averaged across three coronal sections per hemisphere taken at different levels across the anterior–posterior extent of the HCRT field (AP ~ -2.00, -2.45 and -2.85 mm).

* $P < 0.05$

** $P < 0.01$ compared to vehicle-treated animals.